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An OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 Module Is an Essential Early Component of Chitin-Induced Rice Immunity

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SUMMARY

OsCEBiP, a chitin-binding protein, and OsCERK1, a receptor-like kinase, are plasma membrane (PM) proteins that form a receptor complex essential for fungal chitin-driven immune responses in rice. The signaling events immediately following chitin perception are unclear. Investigating the spatiotemporal regulation of the rice small GTPase OsRac1, we find that chitin induces rapid activation of OsRac1 at the PM. Searching for OsRac1 interactors, we identified OsRacGEF1 as a guanine nucleotide exchange factor for OsRac1. OsRacGEF1 interacts with OsCERK1 and is activated when its C-terminal S549 is phosphorylated by the cytoplasmic domain of OsCERK1 in response to chitin. Activated OsRacGEF1 is required for chitin-driven immune responses and resistance to rice blast fungus infection. Further, a protein complex including OsCERK1 and OsRacGEF1 is transported from the endoplasmic reticulum to the PM. Collectively, our results suggest that OsCEBiP, OsCERK1, OsRacGEF1, and OsRac1 function as key components of a "defensome" critically engaged early during chitin-induced immunity.

INTRODUCTION

Plants are static organisms constantly challenged by pathogenic microorganisms. Lacking an adaptive immune system, plants instead rely on innate immunity (Jones and Dangl, 2006). The perception of microbe-associated molecular patterns (MAMPs) by host pattern recognition receptors (PRRs) is important for the initiation step of innate immunity. Host perception of MAMPs induces a rapid defense response, designated MAMP-triggered immunity (MTI), that includes calcium influx, production of reactive oxygen species (ROS), and induction of defense-related genes (Boller and Felix, 2009). Virulent pathogens are able to suppress MTI by expressing pathogen effectors. To counter the activity of pathogen effectors, resistance (R) proteins that act as intracellular receptors for the perception of pathogen effectors activate effector-triggered immunity (ETI) (Jones and Dangl, 2006; Segonzac and Zipfel, 2011). These two layers of defense constitute the plant innate immune system.

Chitin, found in pathogenic and nonpathogenic fungi, is one of the best-studied MAMPs, and chitin signaling has been analyzed extensively in rice and *Arabidopsis* (Gust et al., 2012). Two lysin motif (LysM)-containing plasma membrane proteins, OsCEBiP and OsCERK1, are essential for chitin signaling (Kaku et al., 2006; Shimizu et al., 2010). OsCEBiP is a receptor-like protein with no intracellular domain and binds chitin, whereas OsCERK1 is a receptor-like kinase and does not bind chitin. These two proteins form a receptor complex that transduces the chitin signal to downstream components for immune responses (Kaku et al., 2006; Shimizu et al., 2010; Shinya et al., 2012). In *Arabidosis*, however, AtCERK1 directly binds chitin (Iizasa et al., 2010; Petutschnig et al., 2010; Liu et al., 2012), and CEBiP-like proteins are not involved in chitin signaling (Shinya et al., 2012; Wan et al., 2012). Interestingly, in rice and *Arabidopsis*, both CERK1 receptor kinase and CEBiP-like receptor proteins seem to be involved in signaling induced by bacterial MAMPs, suggesting a general role for CERK1 and CEBiP-like proteins as components of receptor complexes for MAMPs (Willmann et al., 2011; Liu et al., 2012; Gust et al., 2012). Furthermore, fungal pathogens secrete LysM-containing effector proteins that suppress plant immunity by binding chitin oligomers to prevent their recognition by plant receptors (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012).

Another rice PRR, XA21, recognizes the sulfated peptide Ax21, which is found in all *Xanthomonas* species (Lee et al., 2009). In the absence of ligand, XB24 binds XA21 and promotes phosphorylation of Ser/Thr sites on XA21 (Chen et al., 2010b). Upon ligand recognition, XB24 dissociates from XA21, leading to activation of this receptor. In *Arabidopsis*, FLAGELLIN SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR) are receptors for bacterial flagellin and elongation factor Tu (EF-Tu), respectively (Gómez-Gómez and Boller, 2002; Segonzac and Zipfel, 2011). Although a number of proteins interacting with plant

receptors and coreceptors for MAMPs have been isolated, the molecular mechanisms involved in downstream signaling are still unclear (Chen and Ronald, 2011; Segonzac and Zipfel, 2011; Gust et al., 2012). Recently, the receptor-like cytoplasmic kinase OsRLCK185 was identified as an essential immediate downstream signaling partner of OsCERK1 (Yamaguchi et al., 2013).

The Rac/Rop small GTPases constitute a plant-specific Rho subfamily and participate in diverse signal transduction processes, including defense, pollen tube growth, root hair development, ROS production, and hormone responses (Berken, 2006; Yang and Fu, 2007; Kawano et al., 2010b). The rice small GTPase OsRac1 is involved in MTI induced by chitin and sphingolipids that are derived from fungal pathogens (Ono et al., 2001; Suharsono et al., 2002). OsRac1 regulates ROS production through its interaction with the N-terminal region of NADPH oxidase and the ROS scavenger OsMT2b (Kawasaki et al., 1999: Wong et al., 2004, 2007). Furthermore, OsRac1 is activated by the rice NLR family R protein Pit and is required for Pit-mediated resistance against rice blast fungus (Kawano et al., 2010a), indicating that OsRac1 is involved in both MTI and ETI. We have proposed that OsRac1 forms a protein network, termed the defensome network, that regulates rice innate immunity. This network includes OsCERK1, the heat shock protein 90 (Hsp90), Hsp70, the cochaperone Hop/Sti1a, the scaffold protein OsRACK1, the lignin biosynthesis enzyme OsCCR1, and MAPK3 and MAPK6 (Lieberherr et al., 2005; Kawasaki et al., 2006; Thao et al., 2007; Nakashima et al., 2008; Chen et al., 2010a; Kim et al., 2012). Although these studies indicate that OsRac1 is a key regulator of rice immunity, the molecular mechanisms by which OsRac1 is activated by MAMPs are not known.

In yeast and mammals, guanine nucleotide exchange factors (GEFs) control the activity of Rho GTPases. In plants, a family of plant-specific Rac/RopGEFs, containing the highly conserved plant-specific Rac/Rop nucleotide exchange factor (PRONE) domain for GEF activity and variable N- and C-terminal regions, are recognized (Berken et al., 2005). While these PRONE GEFs play important roles in plant development (Duan et al., 2010; Chen et al., 2011; Zhang and McCormick, 2007), their involvement in plant defense is unknown. Recently, OsSWAP70A, a new type of plant GEF having a DH motif homologous to human SWAP70 GEFs for Rho GTPases, has been identified in rice (Yamaguchi et al., 2012); however, its function in disease resistance remains to be studied.

In this study, we identified a PRONE GEF for OsRac1, termed OsRacGEF1. We show that OsRacGEF1 is rapidly activated by chitin at the plasma membrane (PM) of rice cells, where it interacts with OsCERK1 and regulates resistance to rice blast fungus. We provide evidence that the C-terminal S549 of OsRacGEF1 is phosphorylated by the cytoplasmic domain of OsCERK1 upon chitin treatment, thereby activating OsRacGEF1. Thus, we demonstrate that the CEBiP/CERK1-OsRacGEF-OsRac1 module plays a major role for early signaling in rice chitin-induced immunity.

RESULTS

OsRac1 Is Rapidly Activated by MAMPs in Rice Cells

To elucidate the spatio-temporal regulation of OsRac1 activation in MTI, we used the Förster resonance energy transfer (FRET) probe Raichu-OsRac1 (Mochizuki et al., 2001; Wong et al., 2007; Kawano et al., 2010a), an intracellular biosensor that facilitates the monitoring of OsRac1 activation by elicitors in vivo. Raichu-OsRac1 is composed of OsRac1, the CRIB motif of human PAK1, which binds specifically to the GTP-bound form of OsRac1, and CFP and Venus as FRET donor and acceptor (Figures S1A and S1B available online). Raichu-OsRac1 was previously used to reveal an interaction between OsRac1 and RBOH (NADPH oxidase) (Wong et al., 2007) and OsRac1 activation by the rice NB-LRR-type R protein Pit in rice cells (Kawano et al., 2010a).

To analyze OsRac1 activation by MAMPs in rice protoplasts, we transiently expressed Raichu-OsRac1-WT and measured the 525 nm/475 nm emission ratio. In time-lapse analysis, the emission ratio was measured at 3 min intervals after MAMP treatment and used to create FRET images (Figure 1A) in which color is correlated with emission ratio. Fluorescent signals of Raichu-OsRac1 were observed at the PM in the majority of rice cells (Figure 1A). OsRac1 was previously shown to localize to the PM (Ono et al., 2001). We found that not only chitin (Figures 1A and 1B) but also a sphingolipid elicitor (Figures S1C–S1E) induced OsRac1 activation in rice protoplasts. Sphingolipid elicitors are components of the cell membrane of blast fungus and have been shown to induce the accumulation of antimicrobial compounds (phytoalexins), cell death, and increased resistance to infection by compatible pathogens (Umemura et al., 2000; Suharsono et al., 2002; Lieberherr et al., 2005). The emission ratio was low in rice protoplasts expressing Raichu-OsRac1-WT when they were treated with buffer or cellohexaose, an analog of chitin used as a negative control (Figure 1C). The ratio was also low in protoplasts expressing Raichu-OsRac1-DN (dominant negative; Figure S1B) even after chitin treatment but was high in protoplasts expressing Raichu-OsRac1-CA (constitutively active; Figure S1B) in the absence of chitin treatment (Figure 1C). Together, these results show that OsRac1 is rapidly activated at the PM of rice protoplasts after chitin or sphingolipid elicitor treatment.

Identification of OsRacGEF1 as an Activator of OsRac1

To identify GEF(s) for OsRac1, we performed yeast two-hybrid screening, using as a bait a mutant OsRac1, OsRac1 D125N, which is analogous to a Ras mutant (D119N) having lower nucleotide affinity and higher GEF affinity (Cool et al., 1999). We identified one PRONE-type GEF, OsRacGEF1, as a candidate GEF for OsRac1 (Table S1). The rice genome harbors 11 genes encoding PRONE domain proteins (Figure S2A), which have a highly conserved PRONE domain composed of C1, C2, and C3 subdomains and variable N and C termini (Figure S2B).

To study the function of OsRacGEF1, we first examined its interaction with OsRac1 by yeast two-hybrid assays. The results showed that wild-type and OsRac1 D125N interacted with OsRacGEF1 (Figure 2A and Figure S2C). To localize the OsRac1-binding region of OsRacGEF1, we used truncated fragments of OsRacGEF1 for the assays and found that the PRONE domain of OsRacGEF1, but not the N-terminal region (aa 1–84) or C-terminal region (aa 457–561), interacts with OsRac1 (Figure 2A). These results are similar to those for *Arabidopsis* RopGEF8, which binds AtRop4 through its PRONE domain (Thomas et al., 2007). To investigate how the nucleotide-bound state of OsRac1 influences its interaction with OsRacGEF1, we

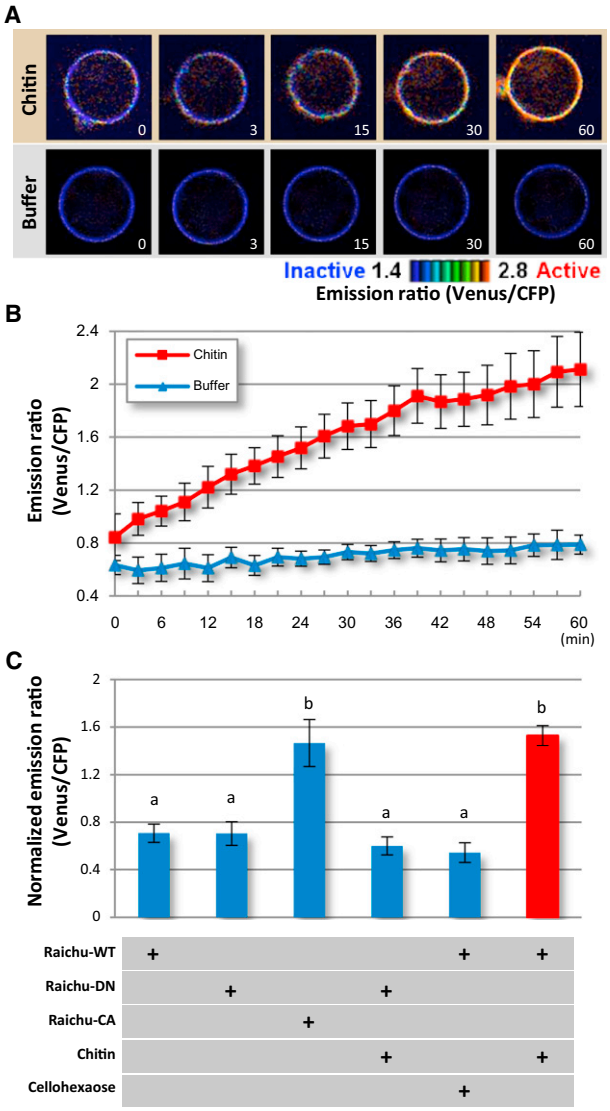

Figure 1. Chitin Induces OsRac1 Activation in Rice Protoplasts

(A) OsRac1 activation during MTI. Time-lapse imaging of Raichu-OsRac1-WT expressed in rice protoplasts. After chitin treatment, emission ratios were measured at 3 min intervals; measurement times (in min) are shown for each image. Eight colors from red to blue represent the observed range of Venus/CFP ratios.

(B) Emission ratios (Venus/CFP) of Raichu-OsRac1-WT. Red, 0.5 μg/ml chitin treatment; blue, W5 buffer treatment.

(C) Average FRET ratios of rice protoplasts transformed with Raichu-OsRac1-WT, Raichu-OsRac1-CA, or Raichu-OsRac1-DN. The ratios were recorded 30–60 min after treatment with chitin, cellohexaose, and buffer treatment. For normalization, crossover was calculated with the CFP fluorescence through the Venus channels. Values marked by the same letters are not significantly different from each other.

Error bars indicate the SE ($p < 0.01$; $n > 20$). See also Figure S1.

performed in vitro binding assays using bacterially produced OsRacGEF1-His and GST-OsRac1. The results showed that OsRac1 interacts directly with OsRacGEF1 in a nucleotide-independent manner (Figure 2B).

We next examined the intracellular localization of OsRacGEF1 using fluorescent probes. Rice protoplasts were cotransformed with OsRacGEF1-Venus, an endoplasmic reticulum (ER) marker, and a PM marker in rice protoplasts and were analyzed under a microscope. We confirmed that fusion of the C-terminal tag of Venus to OsRacGEF1 did not affect the latter's function in defense gene activation (Figure S2D). OsRacGEF1-Venus localized mainly to the cytoplasm, but signals were also detected at the ER and the PM (Figure 2C). When rice protoplasts were transformed with OsRacGEF1-Venus together with CFP-OsRac1-WT, the two molecules clearly colocalized at the PM (Figure 2C). We further confirmed the interaction between OsRac1 and OsRacGEF1 in vivo using bimolecular fluorescence complementation (BiFC) assays (Hu et al., 2002; Chen et al., 2010a). To quantify the interactions in BiFC assays, we measured the frequency of reconstituted Venus-positive protoplasts in each combination of constructs. When OsRac1 tagged with the N-terminal domain (aa 1–154) of Venus (Vn-OsRac1-WT, Vn-OsRac1-DN, and Vn-OsRac1-CA) and OsRacGEF1 tagged with the C-terminal domain (aa 155–238) of Venus (OsRacGEF1-Vc) were coexpressed in rice protoplasts, Venus fluorescence was clearly detected at the PM in all of these combinations (Figure 2D). Together, these results demonstrate that OsRacGEF1 interacts with OsRac1 both in vitro and in vivo. Since all of the interaction studies using fluorescent proteins in rice protoplasts involved constructs having the 35S promoter, we analyzed *OsCERK1-Venus* expression under the native *OsCERK1* promoter and found that both expression level and localization were essentially the same as those with the 35S-*OsCERK1-Venus* construct (Figure S2E). These results indicate that 35S-driven constructs are suitable for image analysis with rice protoplasts.

To further characterize OsRacGEF1, we purified the protein as a recombinant GST fusion protein and determined its in vitro GEF activity. The time-dependent dissociation of GDP from OsRac1 was enhanced by the PRONE domain of OsRacGEF1 relative to full-length OsRacGEF1 (Figure 2E). To confirm these findings in vivo, we monitored activation of OsRac1 by OsRacGEF1 using Raichu-OsRac1. The emission ratio was low in rice protoplasts cotransformed with Raichu-OsRac1 and empty vector. It was higher when the protoplasts were cotransformed with Raichu-OsRac1 and wild-type OsRacGEF1 (Figure 2F). Moreover, the PRONE domain alone enhanced the emission ratio to a greater extent than did wild-type OsRacGEF1. These results are consistent with those of the in vitro GEF assays. Because fluorescent signals were detected at the PM of rice protoplasts, we conclude that OsRac1 activation by the PRONE domain of OsRacGEF1 occurs at the PM of rice cells.

OsRacGEF1 Is Involved in Chitin-Triggered Immune Responses and Resistance to Rice Blast Infection

To examine the function of OsRacGEF1 in rice immunity, we produced transgenic rice plants in which *OsRacGEF1* expression was silenced by RNA interference (RNAi; Figure S3A). In *OsRacGEF1* RNAi transgenic plants, *OsRacGEF1* expression was strongly reduced (Figure 3A). RT-PCR analyses of *OsRacGEF1* messenger RNA extracted from the leaves of transgenic rice plants showed that, among the 11 PRONE domain protein family members, *OsRacGEF1* expression was uniquely suppressed (Figure S3B). Since we were not able to generate cell lines expressing *OsRacGEF1* RNAi, roots of *OsRacGEF1*

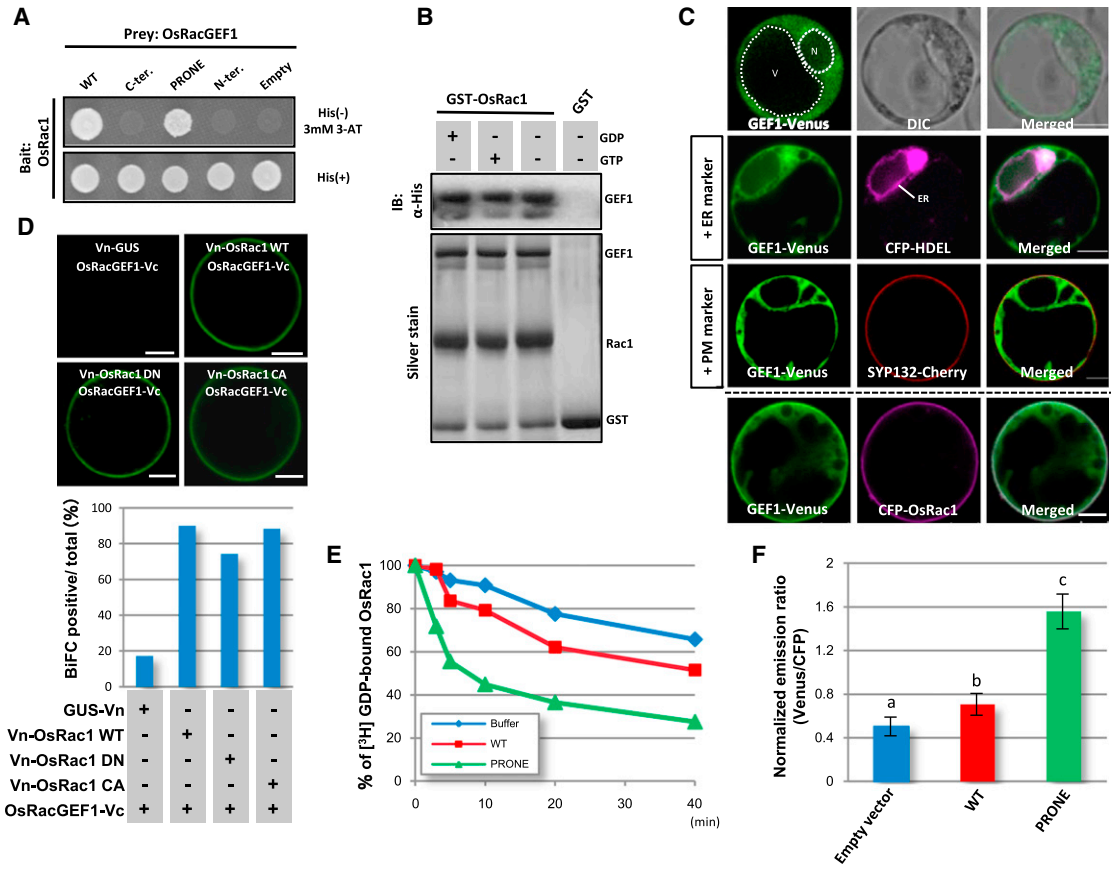

Figure 2. OsRacGEF1 Functions as a GEF for OsRac1

(A) Yeast two-hybrid assays of OsRacGEF1 and OsRac1. Yeast growth on selective plates without histidine [His(–)] and with 3 mM aminotriazole (3-AT) indicates a positive interaction.

(B) In vitro binding assays of OsRacGEF1 and OsRac1 with GTP, GDP, or control (free). Upper image, purified GST-tagged OsRac1 and His-tagged OsRacGEF1 were subjected to this assay with anti-GST beads and detected with anti-His antibody; lower image, silver-stained gel.

(C) Localization of OsRacGEF1 in rice protoplasts. Rice protoplasts were cotransformed with the fluorescent constructs OsRacGEF1-Venus (green), CFP-HDEL (magenta), and SYP132-mCherry (red). Colocalization of CFP-OsRac1 and OsRacGEF1-Venus at the plasma membrane is shown (low). N, V, and ER indicate nucleus, vacuole, and endoplasmic reticulum, respectively.

(D) BiFC assay of OsRacGEF1 and OsRac1 in rice protoplasts. Expression of these genes was driven by the CaMV 35S promoter. The graph (below) quantifies the interaction of Vn-OsRac1 and OsRacGEF1-Vc at the PM. GUS serves as a negative control.

(E) In vitro GEF assay of OsRacGEF1. The effect of OsRacGEF1 on the time-dependent dissociation of GDP from OsRac1 in vitro was detected by [^{3}H]-labeled GDP.

(F) In vivo GEF assay of OsRacGEF1 by Raichu-OsRac1 FRET sensor. The graph shows the average normalized emission ratio of rice protoplasts transformed with the indicated plasmids. The ratios were observed 30–60 min after treatment with chitin. For normalization, crossover was calculated with the CFP fluorescence through the Venus channels. Values marked by the same letters are not significantly different from each other. Error bars represent the SE ($p < 0.05$; $n > 20$). Scale bars in (C) and (D) represent 5 μm. See also Figure S2 and Table S1.

RNAi and wild-type plants were treated with chitin, and changes in the expression of known chitin-induced genes were monitored by quantitative PCR (qPCR). Expression of *PAL1*, *PBZ1*, *Chitinase1*, and *Chitinase3* was strongly suppressed in *OsRacGEF1* RNAi plants compared to the wild-type (Figures 3B–3E). Chitin-induced ROS production was measured by chemiluminescence (Figure 3F). ROS production was strongly suppressed in all three RNAi lines relative to the wild-type. When *OsRacGEF1* RNAi plants were infected with a virulent strain of the rice blast fungus (*Magnaporthe oryzae* strain 2403-1, race 007), they were more susceptible than wild-type plants (Figures 3G–3I). To measure fungal growth in *OsRacGEF1* RNAi plants, we used DNA-based real-time PCR to quantify *M. oryzae* with two sets of primers specific for *M. oryzae Pot2* and rice *ubiquitin* (Figure 3I). The analysis showed that the infection ratio (*MgPot2*/*Osubiquitin*) was higher in *OsRacGEF1* RNAi plants than in wild-type plants at 6 days postinoculation (dpi), indicating that *OsRacGEF1* RNAi plants are more susceptible to blast fungus infection than are wild-type plants. Taken together, these results showed that OsRacGEF1 is involved in chitin-triggered immune responses and plays a role in resistance to blast infection.

OsRacGEF1 Interacts with Pattern Recognition Receptors at the ER and the PM

To examine the possibility that PRRs might transmit signals to OsRac1 through OsRacGEF1, we first tested for interactions

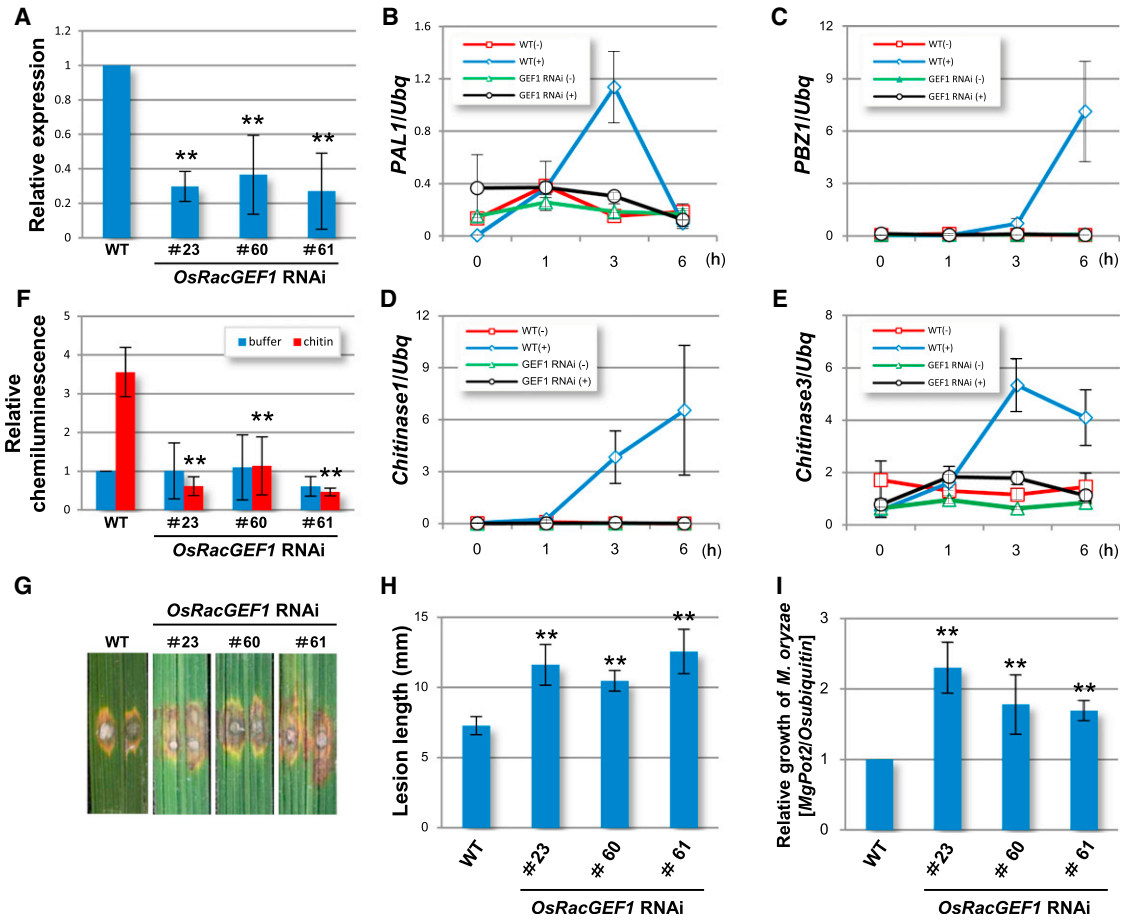

Figure 3. OsRacGEF1 Is Involved in the Chitin-Triggered Immune Response and Rice Blast Resistance

(A) Transcript levels of *OsRacGEF1* were measured by qPCR in *OsRacGEF1* RNAi plants and normalized with endogenous *Ubq*. Data shown are means. The numbers indicate independent RNAi transgenic lines. Relative expression is shown (WT = 1). Error bars indicate the SE (**$p < 0.01$; $n = 3$).

(B–E) Induction of the defense-related genes *PAL1* (B), *PBZ1* (C), *Chitinase1* (D), and *Chitinase3* (E) in *OsRacGEF1* RNAi and WT roots, after chitin treatment (+), was monitored at the indicated time points with qPCR. (–), mock. Data are means ($n = 3$). Error bars indicate the SE.

(F) ROS production in *OsRacGEF1* RNAi roots after treatment with 10 μg/ml chitin. ROS production was quantified with a luminescence image analyzer. Relative chemiluminescence is shown (WT = 1). Error bars indicate the SE (**$p < 0.01$; $n = 3$).

(G–I) Infection assays of *OsRacGEF1* RNAi plants with the compatible *M. oryzae* race 007.

(G) The photographs show typical phenotypes of WT and representative *OsRacGEF1* RNAi plants.

(H) Quantitative analysis of lesions induced by the blast fungus was performed at 6 days postinfection. Relative lesion lengths (WT = 1) are shown with means. Error bars indicate the SE (**$p < 0.01$; $n > 60$).

(I) Growth of the compatible *M. oryzae* race in *OsRacGEF1* RNAi plants. Error bars indicate the SE (**$p < 0.01$; $n > 6$).

See also Figure S3.

between PRRs and OsRacGEF1 using three rice PRRs: OsCERK1, a component of the rice chitin receptor complex; the rice LRR kinase OsFLS2, which is an ortholog of AtFLS2 and functions in flagellin perception (Takai et al., 2008); and XA21. We tested their in vivo interaction with OsRacGEF1 by coimmunoprecipitation (coIP) experiments, using rice protoplasts coexpressing OsRacGEF1-Venus and OsCERK1-FLAG, OsFLS2-FLAG, or XA21-FLAG. The results showed that OsRacGEF1 interacts with both OsCERK1 and OsFLS2 in a MAMP-independent manner (Figures 4A and 4B). However, no interaction was detected between OsRacGEF1 and XA21 (Figure S4A). Chitin treatment did not affect the OsCERK1-OsRacGEF1 interaction (Figure S4B).

We further examined interactions between OsRacGEF1 and OsCERK1 or OsFLS2 by BiFC assays (Figures 4C and 4D). In rice protoplasts coexpressing the N-terminal half of Venus fused to OsCERK1 and the C-terminal half fused to OsRacGEF1, the BiFC signal was mainly localized to the PM and the ER (Figure 4C). Similar results were obtained between OsRacGEF1 and OsFLS2 (Figure 4D). We measured the frequency of reconstituted Venus-positive protoplasts in each combination of constructs and found interactions between OsRacGEF1 and both OsCERK1 and OsFLS2 (Figures 4C and 4D, right). Moreover, we performed yeast two-hybrid assays with the C-terminal domain of OsRacGEF1 and the cytoplasmic domain of OsCERK1 (Figure 4E) and found that they interact with each

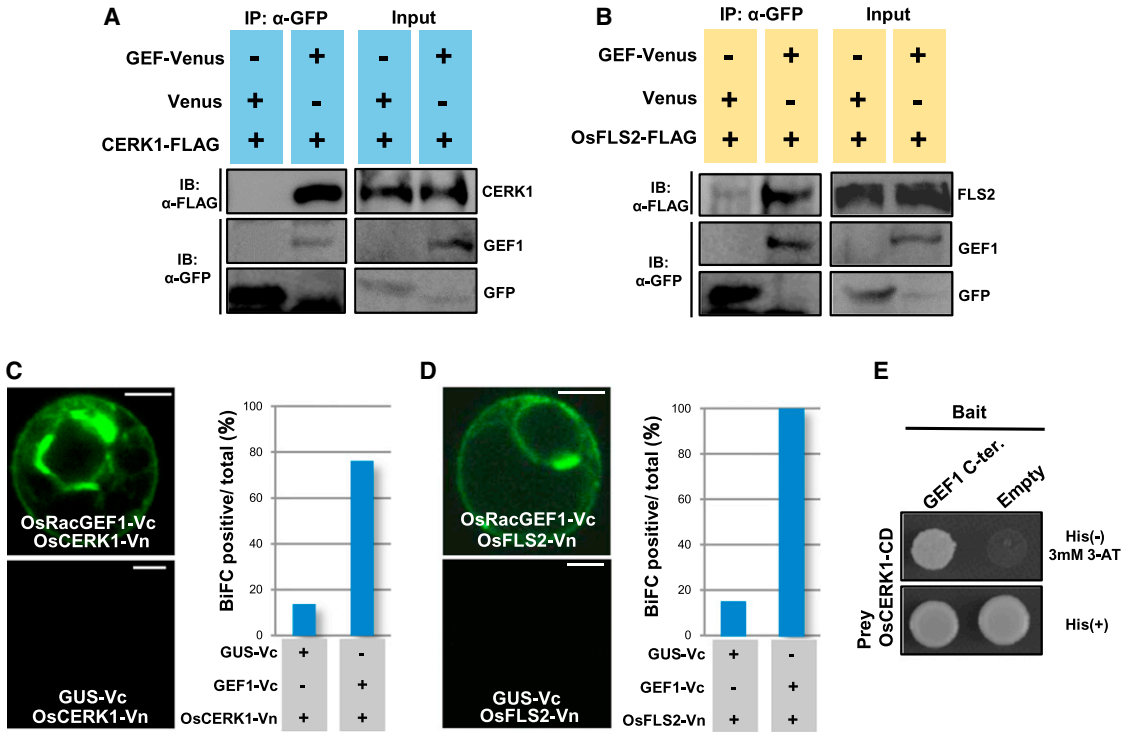

Figure 4. Interaction of OsRacGEF1 with PRRs in Rice

(A and B) OsRacGEF1-Venus and OsCERK1-FLAG or OsFLS2-FLAG were transiently coexpressed in rice protoplasts. CoIP was carried out with anti-GFP (IP, α-GFP), and the proteins were analyzed by western blot with anti-GFP antibody (IB, α-FLAG).

(C and D) BiFC assays of OsRacGEF1 and PRRs. OsRacGEF1-Vc, and OsCERK1-Vn, or OsFLS2-Vn, was used. GUS-Vc was used as a negative control. The graphs to the right are quantitative evaluations of each BiFC pair. Scale bars represent 5 μm. Shown in (D) are yeast two-hybrid assays of the C-terminal region of OsRacGEF1 and the cytoplasmic domain (CD) of OsCERK1. Yeast growth on selective plates without histidine [His (–)] and with 3 mM aminotriazole (3-AT) indicates a positive interaction.

See also Figure S4.

other. Interestingly, the cytoplasmic domain of OsFLS2 interacted with the PRONE domain of OsRacGEF1, but not with its C-terminal domain (Figure S4C). Together, these results demonstrated that OsCERK1 and OsFLS2 interact with OsRacGEF1 in vivo and in vitro.

Phosphorylation of OsRacGEF1 Serine 549 Is Critical for GEF Activity and Rice Immunity

In the *Arabidopsis* PRONE-type GEF AtRopGEF12, mutation of the conserved serine 510 to a phosphomimicking residue has been shown to overcome an autoinhibition of GEF function by the C-terminal region (Zhang and McCormick, 2007). The C-terminal region of OsRacGEF1 interacts with the PRONE domain (Figure S2F), and full-length OsRacGEF1 showed lower GEF activity than did the PRONE domain alone (Figures 2E and 2F). These results indicate that the C-terminal region of OsRacGEF1 inhibits GEF activity, as shown for AtRopGEF12. Therefore, we examined whether phosphorylation of C-terminal serine residues in OsRacGEF1 is important for releasing the C-terminal inhibition in vivo. We generated full-length OsRacGEF1 with point mutations at the two conserved serine residues S480 and S549, to phosphomimic aspartic acid (D), using site-directed mutagenesis. We tested whether these OsRacGEF1 mutants induced activation of OsRac1 in rice protoplasts using the Raichu-OsRac1 FRET sensor (Figure 5A). The emission ratio was high in protoplasts transformed with OsRacGEF1 S549D or the PRONE domain alone, but low in protoplasts transformed with OsRacGEF1 WT or OsRacGEF1 S480D, indicating that phosphorylation of S549 is important for OsRac1 activation.

To investigate whether S549 phosphorylation affects rice immunity, we produced transgenic rice cell cultures and plants overexpressing (OX) *OsRacGEF1* S549A (Figure S3A). *OsRacGEF1* expression was significantly increased in plant numbers 19 and 21, but not in plant number 8 (Figure S3C). Transgenic cell lines were treated with chitin, and chitin-induced defense-related gene expression was monitored by qPCR. Expression of *PAL1*, *PBZ1*, *Chitinase1*, and *Chitinase3* was strongly reduced in *OsRacGEF1 S549A* OX cell lines compared to wild-type cell lines (Figures 5B–5E). Moreover, infection assays with the virulent strain of rice blast fungus revealed that *OsRacGEF1 S549A* OX plant numbers 19 and 21 were more susceptible than wild-type plants to rice blast infection, but that line number 8, which did not overexpress *OsRacGEF1* S549A, was not (Figures 5F–5H). Together, these results indicate that OsRacGEF1 S549 is critical for chitin-induced immunity and resistance to blast fungus infection.

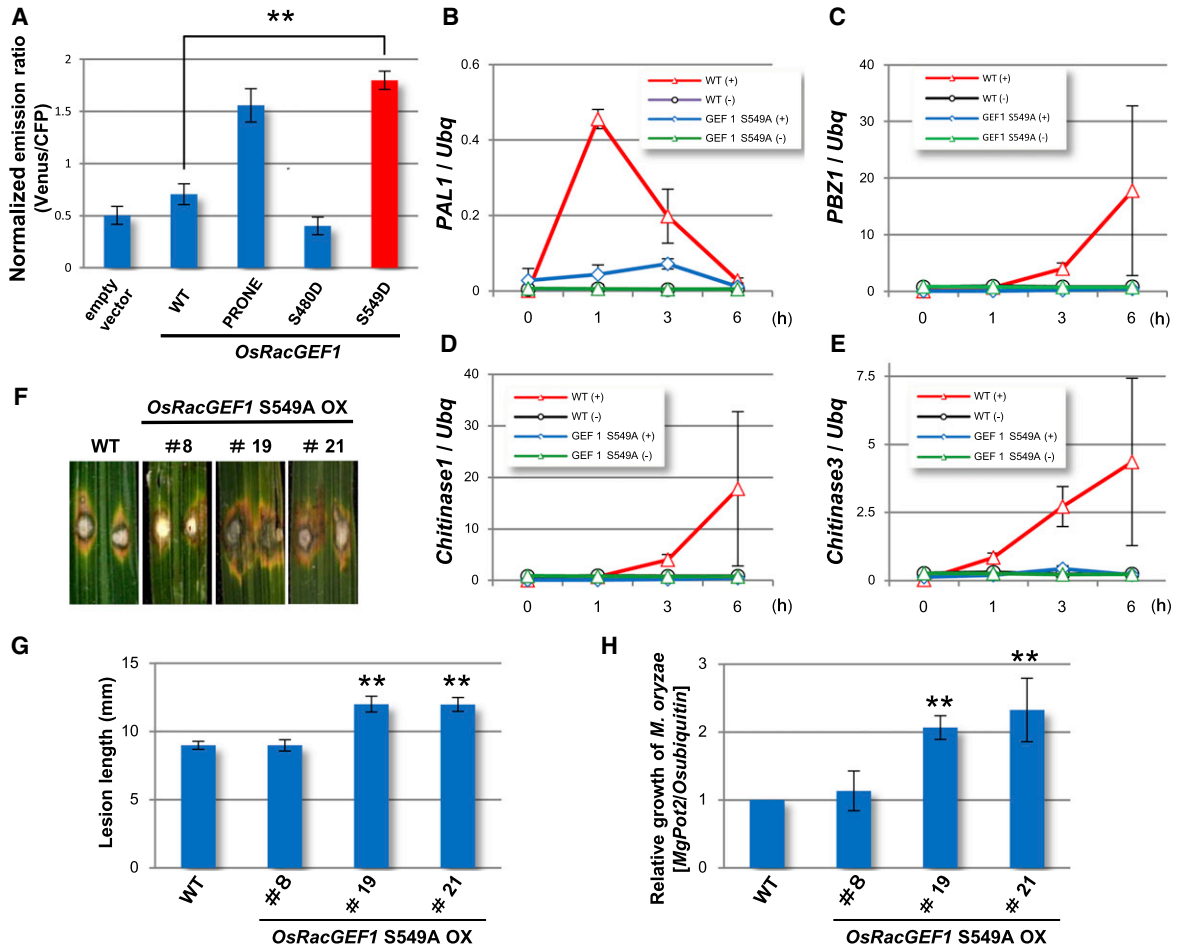

Figure 5. Phosphorylation of OsRacGEF1 S549 Is Critical for GEF Activity and Rice Immunity

(A) In vivo GEF assay of OsRacGEF1 S549D by Raichu-OsRac1 FRET sensor. The graph shows the average ratio of rice protoplasts transformed with the indicated plasmids. The ratios were measured 30–60 min after treatment with chitin. Error bars indicate the SE (**$p < 0.01$; $n > 20$).

(B–E) Induction of the defense-related genes *PAL1* (B), *PBZ1* (C), *Chitinase1* (D), and *Chitinase3* (E) in OsRacGEF1 S549A and WT cultured cells, after chitin treatment (+), was monitored at the indicated time points using qPCR. (–), mock. Data are means ($n = 3$). Error bars indicate the SE.

(F–H) Infection assays of *OsRacGEF1* S549A OX plants treated with the compatible *M. oryzae* race 007.

(F) The photographs show typical phenotypes of WT and representative *OsRacGEF1* S549A OX plants.

(G) Quantitative analysis of lesions induced by the blast fungus was performed at 6 dpi. Lesion lengths are shown with means. Error bars indicate the SE (**$p < 0.01$; $n > 60$).

(H) Relative growth (WT = 1) of *M. oryzae* on susceptible rice cultivars. Error bars indicate the SE (**$p < 0.01$; $n > 6$).

OsRacGEF1 S549 Is Phosphorylated by OsCERK1

To test whether OsRacGEF1 S549 is phosphorylated upon chitin treatment in rice cells, we performed mass spectrometric analysis of OsRacGEF1 immunoprecipitated from chitin-treated or nontreated protoplasts (Figure 6A, 6B, S5A, and S5B). S549 was the only phosphorylated residue in the C-terminal domain (aa 457–561) of OsRacGEF1 after chitin treatment (Figure 6A). Without chitin treatment, no phosphrylation of S549 was detected (Figure S5B). Next, to examine whether OsRacGEF1 was phosphorylated by the intracellular protein kinase domain of OsCERK1, we incubated the C-terminal region of OsRacGEF1 with the intracellular domain of OsCERK1 (CD) in the presence of ^{32}P-labeled ATP. The results indicated that the C-terminal region of OsRacGEF1 was phosphorylated (Figure 6C), whereas the kinase-dead (KD) CD mutant K352N had no such effect (Figure 6C). These results indicate that OsRacGEF1 is a direct substrate of OsCERK1. To determine whether S549 is phosphorylated by OsCERK1, we tested phosphorylation of the mutant OsRacGEF1 S549A. The lower level of phosphorylation observed in OsRacGEF1 S549A than in the wild-type suggests that S549 is phosphorylated by OsCERK1. Furthermore, because the S549 mutant interacted with OsRac1 as wild-type OsRacGEF1 did, S549 phosphorylation does not affect the interaction between OsRacGEF1 and OsRac1 (Figure S5B). Together, these results indicate that phosphorylation of OsRacGEF1 S549 by the protein kinase domain of OsCERK1 results in activation of OsRaGEF1, thereby leading to OsRac1 activation.

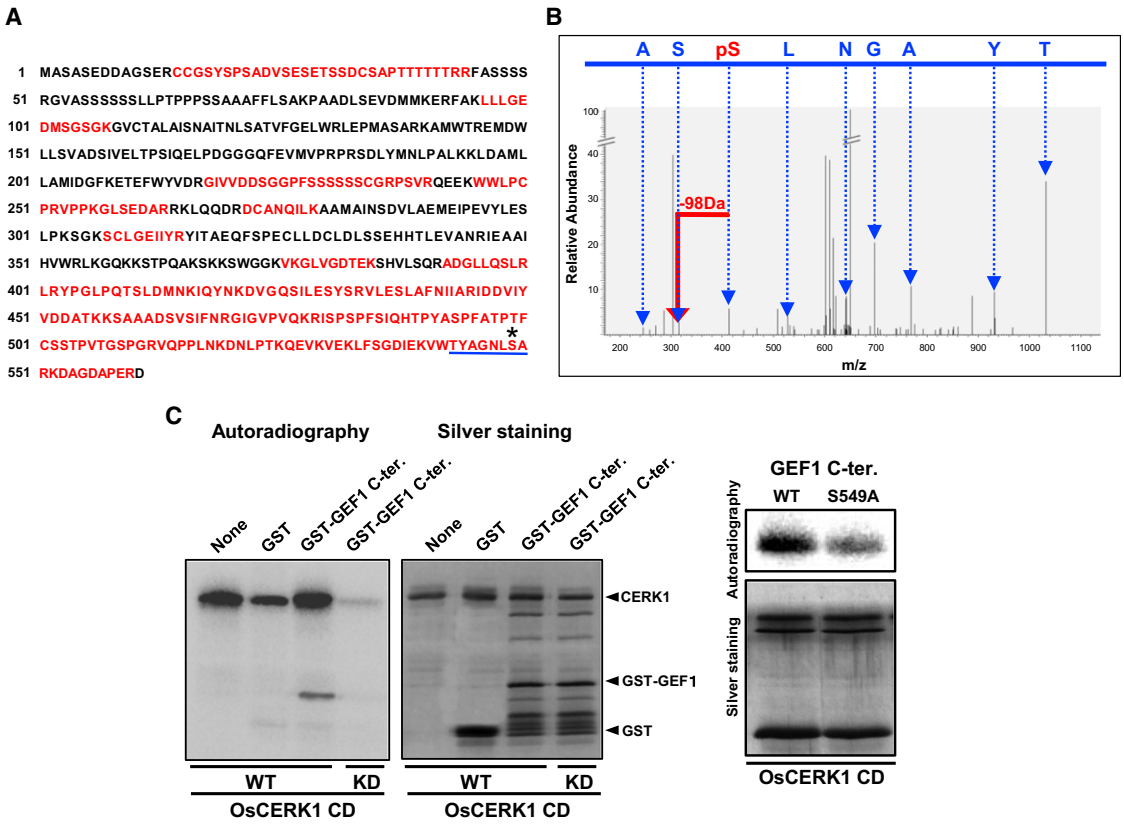

Figure 6. Phosphorylation of OsRacGEF1 by OsCERK1 In Vitro and Chitin-Dependent Phosphorylation of OsRacGEF1 In Vivo

(A and B) Phosphorylation of OsRacGEF1 in the presence of chitin. Peptides were analyzed by liquid chromatography-tandem mass spectrometry. The identified phosphoseryl residue is denoted as pS.

(A) Amino acid sequence of OsRacGEF1. Matched peptides are shown in red. The asterisk indicates the phosphorylation site.

(B) Mass spectrometry spectrum identifying in vivo phosphorylation sites of OsRacGEF1 peptides.

(C) Phosphorylation analysis of the C-terminal region (C-ter.) of OsRacGEF1 (left panel), and the C-terminal region of OsRacGEF1 S549A and the cytoplasmic domain (CD) of OsCERK1 (right panel). GST alone, GST-CERK1(CD), and the C-terminal region of OsRacGEF1 were expressed in *E. coli* and purified. These samples were incubated in a reaction buffer containing [γ-^{32}P] at 30°C for 20 min.

See also Figure S5.

OsRacGEF1 Is Transported from the ER to the PM through a Vesicle Trafficking Pathway

Subcellular trafficking of PRRs is important for plant immunity (Beck et al., 2012). We showed previously that OsCERK1 is transported from the ER to the PM through a Sar1-dependent vesicle trafficking pathway (Chen et al., 2010a). A constitutively active *Arabidopsis* Sar1 (CA-AtSar1) mutant inhibits transport of the Golgi membrane protein AtRer1B from the ER to the Golgi (Takeuchi et al., 2000). Since OsRacGEF1 interacts with OsCERK1 at the ER in rice protoplasts (Figure 4C), we examined whether localization of OsRacGEF1 is also regulated by the COPII-mediated ER-to-Golgi trafficking pathway. We cotransformed rice protoplasts with OsRacGEF1-CFP, GFP-AtRer1B, and either WT-AtSar1 or CA-AtSar1 and examined their subcellular localizations. OsRacGEF1-CFP predominantly localized to the PM, the cytosol, and the ER, while AtRer1B-GFP was found in Golgi-like organelles of protoplasts cotransformed with WT-AtSar1 (Figures 7A and 7B). In contrast, in cells cotransformed with CA-AtSar1, the AtRer1B-GFP signal was restricted to the ER. Similarly, OsCERK1-CFP and OsRacGEF1-CFP signals were only detected in the ER (Figures 7A and 7B). To study whether OsRac1, OsRacGEF1 and OsCERK1 form a complex, we performed coIP experiments (Figure S4D). Results suggest that OsRac1 can possibly form a complex with OsCERK1 and OsRacGEF1 in vivo. These and our previous results (Chen et al., 2010a) suggest that OsRacGEF1 is transported, together with OsCERK1 and chaperones such as Hsp90, Hsp70, and Hop/Sti1, from the ER to the PM through a Sar1-dependent vesicle trafficking pathway and that they form a complex with OsRac1 at the PM.

DISCUSSION

Rapid Activation of OsRac1 at the PM in MAMP-Triggered Immunity

Although previous studies have established that OsRac1 is required for MTI, the spatiotemporal dynamics of OsRac1 activation during the defense response has not been investigated. Here we showed that OsRac1 is activated at the PM of rice protoplasts within 3 min after chitin treatment (Figures 1A–1C).

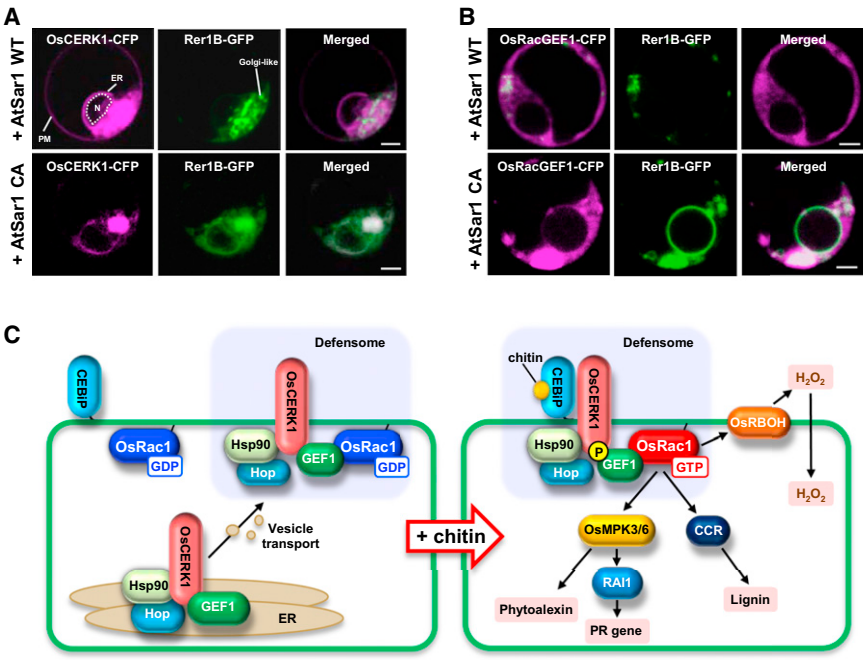

Figure 7. OsRacGEF1 Is Transported from the ER to the PM with OsCERK1 through a Vesicle Trafficking Pathway

(A and B) OsCERK1-CFP (A) and OsRacGEF1-CFP (B) localization patterns in the presence of WT- or CA-AtSar1. Rice protoplasts were cotransformed with OsCERK1-CFP (A) or OsRacGEF1-CFP (B) and either WT-AtSar1-Rer1B-GFP or CA-AtSar1-Rer1B-GFP. Rer1B was used as a Golgi marker. Scale bars represent 5 μm. N, PM, and ER indicate nucleus, plasma membrane, and endoplasmic reticulum, respectively.

(C) Working model for intracellular transport of OsRacGEF1 and chitin-induced rice immunity. OsRacGEF1 interacts with OsCERK1 and chaperones at the ER, and the complex is transported to the PM, where it forms the defensome complex with OsRac1 (left). Upon chitin perception by OsCEBiP, OsCEBiP dimerizes with OsCERK1 and the OsCERK1 kinase phosphorylates S549 of OsRacGEF1, which activates OsRac1. Activated OsRac1 induces various downstream immune responses, leading to disease resistance.

Chitin is one of the most common MAMPs, since it is highly conserved in fungi (Boller and Felix, 2009; Chen and Ronald, 2011; Gust et al., 2012). Sphingolipid elicitors induce OsRac1 activation in a similar manner (Figures S1C–S1E). Sphingolipids are components of the cell membrane of rice blast fungus, and they induce a variety of immune responses in rice, including phytoalexin synthesis, MAPK activation, and PR gene expression (Umemura et al., 2000; Suharsono et al., 2002; Lieberherr et al., 2005). However, receptors for sphingolipid elicitors are not known. Our results suggest that OsRac1 activation is a general early response against various microbial pathogens of rice.

It has been shown that phosphorylation of the plant LRR kinase FLS2 and BAK1 is rapid, within 15 s of stimulation with flagellin (Schulze et al., 2010). Moreover, increases in cytoplasmic Ca^{2+} concentration and OsMAPK6 activation occur within 5 min after chitin treatment (Kishi-Kaboshi et al., 2010). Similarly, analysis of Rac activation in nerve growth factor (NGF)-stimulated rat cells with a Raichu-Rac FRET probe revealed that Rac1 is activated within 2 min after NGF treatment (Nakamura et al., 2005). Therefore, the rapidity with which OsRac1 is activated by MAMPs in rice cells is consistent with that in other signaling systems in plants and mammalian cells.

OsRacGEF1 Functions Downstream of OsCERK1 in Rice MTI

It was previously shown that the PRONE GEFs play an important role as exchange factors for the Rac/Rop GTPase family (Berken et al., 2005). In this study, we identified the PRONE-type OsRacGEF1 as an OsRac1-interacting protein in a yeast two-hybrid screen (Figure S2C and Table S1), and pull-down and BiFC assays confirmed their interaction (Figures 2B and 2C). Although the pull-down assays showed that OsRacGEF1 binding to OsRac1 is nucleotide independent (Figure 2B), it is possible that OsRacGEF1 associates with OsRac1-GDP (Figures 2E and 2F). In *OsRacGEF1* RNAi plants, chitin-induced expression of *PAL1*, *PBZ1*, *Chitinase1*, and *Chitinase3* and ROS production were strongly suppressed (Figures 3B–3F), and they were more susceptible than wild-type plants to rice blast infection (Figures 3G–3I). The levels of suppression of both chitin-induced defense gene expression and ROS production in *OsRacGEF1* RNAi plants were similar to those found in RNAi plants of *OsCEBiP* (Kaku et al., 2006) and *OsCERK1* (Shimizu et al., 2010), suggesting that OsRacGEF1 is a major GEF for OsRac1 during rice chitin signaling. Furthermore, OsFLS2 also interacts with OsRacGEF1 in a manner similar to that of OsCERK1. Interestingly, however, Xa21 does not interact with OsRacGEF1, suggesting that the components of signaling downstream of Xa21 may be different from those of OsCERK1 and OsFLS2. In this regard, recent identification of the receptor-like cytoplasmic kinase OsRLCK185, which is phosphorylated by OsCEK1, is interesting (Yamaguchi et al., 2013). It connects with a MAP kinase cascade. Therefore, two signaling pathways, OsRacGEF1-OsRac1 and OsRLCK185-OsMAPKs, seem to function immediate downstream of OsCERK1 in rice chitin-induced immunity.

OsSWAP70A, a homolog of a human DH domain-containing GEF for Rho GTPase, was recently isolated (Yamaguchi et al., 2012). It displays in vitro GEF activity toward OsRac1, and *SWAP70A* RNAi cells showed reduced defense gene expression and ROS production upon chitin treatment. However, *OsSWAP70A* RNAi plants showed no reduction in resistance to rice blast infection (T.K., unpublished data), and OsSWAP70A is localized to endosomes and interacts there with OsCERK1 (A.A., unpublished data). Furthermore, the levels of suppression of both chitin-induced defense gene expression and ROS production in *OsRacGEF1* RNAi plants were higher than those in *OsSWAP70A* RNAi plants. These results suggest that

OsRacGEF1 is a primary GEF for OsRac1, functioning at the PM during MTI.

While plant PRRs have long been studied, their downstream signaling pathways are unclear (Boller and Felix, 2009; Segonzac and Zipfel, 2011; Schwessinger and Ronald, 2012). OsRacGEF1 interacts with OsCERK1 at the PM (Figures 4A and 4C). Since the OsCERK1-OsRaGEF1 interaction is chitin independent (Figure 4A), it is likely that when OsCEBiP perceives chitin, it interacts with the OsCERK1-OsRacGEF1complex at the PM. Our BiFC and coIP experiments indicate that OsRacGEF1 also interacts with the flagellin receptor OsFLS2 (Figures 4B and 4D) (Takai et al., 2008).

Trafficking of OsRacGEF1 with OsCERK1

Hop/Sti1 and Hsp90 are required for efficient transport of OsCERK1 from the ER to the PM via a pathway that depends on Sar1 in rice (Chen et al., 2010a). The cytoplasmic and PM localization of the PRONE GEF family has recently been reported (Zhang and McCormick, 2007; Chen et al., 2011). Our analysis of OsRacGEF1-Venus showed that it is localized mainly in the cytoplasm, the PM, and the ER (Figure 2A). Furthermore, BiFC analysis revealed that OsRacGEF1 interacts with PRRs at the ER and the PM (Figure 4B). These results, together with our previous study (Chen et al., 2010a), suggest that OsRacGEF1 is part of a protein complex consisting of OsCERK1 and Hsp90 and other chaperones and that the complex is transported from the ER to the PM.

OsCERK1, OsRacGEF1, and OsRac1 Are Key Components of the Defensome in Rice Immunity

OsCERK1 interacts with CEBiP, which binds chitin with its extracellular LysM motifs (Kaku et al., 2006; Shimizu et al., 2010). However, events after chitin perception have not yet been studied. In *Arabidopsis*, CERK1 forms a homodimer after chitin perception, and this dimerization is critical for its activation (Liu et al., 2012), but CEBiP homologs are not involved in chitin signaling (Shinya et al., 2012; Wan et al., 2012). Downstream components phosphorylated by CERK1 in rice or *Arabidopsis* remain unidentified. In *Arabidopsis* flagellin signaling, FLS2 associates with the LRR receptor kinase BAK1 in a flagellin-dependent manner (Chinchilla et al., 2007), and intracellular signaling is initiated by transphosphorylation of FLS2 by BAK1 (Schulze et al., 2010).

In this study, we showed that OsRacGEF1 S549 is phosphorylated by the cytoplasmic kinase of OsCERK1 upon chitin treatment (Figures 6 and S5). Furthermore, the S549D mutation in OsRacGEF1 induced OsRac1 activation, as did the OsRacGEF1 PRONE domain. Interestingly, the closest *Arabidopsis* ortholog of OsRacGEF1 is AtRopGEF1; their C-terminal amino acid sequences are highly conserved, and OsRacGEF1 S549 seems to be conserved in AtRopGEF1 (Figure S5D). Collectively, our results suggest that the OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 module plays an important role in chitin-induced immunity. We found that two distinct types of PRR, OsCERK1 and OsFLS2, interact with OsRacGEF1 (Figures 4A and 4B). Although it has not yet been identified, a receptor for sphingolipid elicitor could interact with OsRacGEF1, since OsRacGEF1 activates OsRac1 at a rate similar to that observed in chitin signaling (Figures S1C–S1E).

Our previous studies showed that OsCERK1 forms a protein network, the defensome network, with OsRac1 and Hop/Sti1a-Hsp90-Hsp70 chaperones at the PM (Thao et al., 2007; Wong et al., 2007; Nakashima et al., 2008; Chen et al., 2010a). OsRacGEF1 is also present in this network, and its phosphorylation by OsCERK1 probably occurs there (Figure 7C). Once OsRac1 is activated in response to chitin, it transduces signals to various downstream signaling components: NADPH oxidase (Rboh) for ROS production, CCR1 for lignin synthesis, MAPK cascades, and RAI1 transcription factor all function downstream of OsRac1 in rice MTI (Figure 7C) (Lieberherr et al., 2005; Kawasaki et al., 2006; Wong et al., 2007; Kim et al., 2012). Biochemical characterization of the defensome suggests that it is an ~350 kDa protein complex present in proximity to the plasma membrane, and that it contains OsCERK1, OsRacGEF1, OsRac1, and chaperones (Chen et al., 2010a) (K.S., unpublished data). Our previous finding that OsRac1 is activated by an NB-LRR-type R protein suggests that OsRac1 is also involved in ETI (Kawano et al., 2010a). Whether OsRacGEF1, too, is involved in ETI remains to be studied, but our unpublished study suggests that there are two types of defensome complex for MTI and ETI and that they share several protein components such as OsRac1 and chaperones (K.S., unpublished data). The OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 module identified in this study might therefore be the central component of the defensome specifically involved in rice MTI.

EXPERIMENTAL PROCEDURES

FRET Analysis

In Raichu-OsRac1, the donor and acceptor ñuorophores and the two interacting proteins are all included in the same molecule. Therefore, the molar ratios of the individual units are the same irrespective of expression level. This reduces errors caused by differences in the levels of donor and acceptor ñuorophores (Wong et al., 2007). For making the Raichu-OsRac1 construct, two sequences for localization signals, the posttranslational modification site with a farnesyl moiety, and the polybasic region of the original Raichu-HsRac1 vector (Mochizuki et al., 2001) were replaced by those of OsRac1 (Wong et al., 2007; Kawano et al., 2010a).

Rice protoplasts were transformed with Raichu-OsRac1 using polyethylene glycol (PEG) solution (Chen et al., 2010a). Ten to 12 hr after transformation, the cells were imaged with an Olympus IX-81 inverted microscope with a Yokogawa CSU22 confocal scanner, equipped with an EM-CCD C9100-02 cooled charge-coupled device camera (Hamamatsu Photonics). Raichu-OsRac1 was excited with a 440 nm diode laser (iFLEX 2000, Point Source). The CFP and Venus filters were 480 ± 15 nm and 535 ± 20 nm, respectively. Background fluorescence was subtracted, and FRET efficiency was calculated according to published procedures (Sorkin et al., 2000).

Chitin Treatment and Quantitative Real-Time PCR Analysis

Cultured rice cells, protoplasts, and roots were treated with 10 μg/ml chitin (hepta-N-acetylchitoheptaose; Sigma) or W5 buffer (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES [pH 5.7]) as a negative control and were harvested at the indicated times after treatment. Total RNA was extracted with an RNeasy Plant Mini Kit (QIAGEN) and treated with DNase I (Invitrogen). Complementary DNA (cDNA) was synthesized from 1 mg total RNA with SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed with samples of cDNA and standard plasmids with SYBR Green PCR master mix (Applied Biosystems) and the gene-specific primers listed in Table S2. Data were collected with the ABI PRISM 7000 sequence detection system according to the manufacturer's instructions.

Quantification of ROS

For measurement of ROS, rice roots were cut and placed into white 96-well plates (Greiner Bio-one). The W5 buffer was removed and replaced with fresh

medium containing 0.5 mM L-012 (Wako Chemicals) and 10 μg/ml chitin elicitor (hepta-N-acetylchitoheptaose; Sigma). Chemiluminescence was monitored at 160 min after chitin treatment with a LAS-4000 Mini Luminescent image analyzer (GE Healthcare).

Mass Spectrometric Analysis

Protein bands excised from a Flamingo (BioRad)-stained SDS gel were excised and subjected to in-gel digestion with trypsin (Fujiwara et al., 2009). The digested peptides were loaded onto an L-Column (100 μm internal diameter, 15 cm; CERI) with a Paradigm MS4 HPLC pump (Michrom BioResources) and an HTC-PAL autosampler (CTC Analytics), and the eluted peptides were introduced into a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The tandem mass spectrometry spectra were compared against databases in National Center for Biotechnology Information with the Mascot server. The peak data of phosphopeptides were confirmed manually.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.03.007.

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REFERENCES

Beck, M., Heard, W., Mbengue, M., and Robatzek, S. (2012). The INs and OUTs of pattern recognition receptors at the cell surface. Curr. Opin. Plant Biol. *15*, 367–374.

Berken, A. (2006). ROPs in the spotlight of plant signal transduction. Cell. Mol. Life Sci. *63*, 2446–2459.

Berken, A., Thomas, C., and Wittinghofer, A. (2005). A new family of RhoGEFs activates the Rop molecular switch in plants. Nature *436*, 1176–1180.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. *60*, 379–406.

Chen, X., and Ronald, P.C. (2011). Innate immunity in rice. Trends Plant Sci. *16*, 451–459.

Chen, L., Hamada, S., Fujiwara, M., Zhu, T., Thao, N.P., Wong, H.L., Krishna, P., Ueda, T., Kaku, H., Shibuya, N., et al. (2010a). The Hop/Sti1-Hsp90 chaperone complex facilitates the maturation and transport of a PAMP receptor in rice innate immunity. Cell Host Microbe *7*, 185–196.

Chen, X., Chern, M., Canlas, P.E., Ruan, D., Jiang, C., and Ronald, P.C. (2010b). An ATPase promotes autophosphorylation of the pattern recognition receptor XA21 and inhibits XA21-mediated immunity. Proc. Natl. Acad. Sci. USA *107*, 8029–8034.

Chen, M., Liu, H., Kong, J., Yang, Y., Zhang, N., Li, R., Yue, J., Huang, J., Li, C., Cheung, A.Y., and Tao, L.Z. (2011). RopGEF7 regulates PLETHORA-dependent maintenance of the root stem cell niche in Arabidopsis. Plant Cell *23*, 2880–2894.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature *448*, 497–500.

Cool, R.H., Schmidt, G., Lenzen, C.U., Prinz, H., Vogt, D., and Wittinghofer, A. (1999). The Ras mutant D119N is both dominant negative and activated. Mol. Cell. Biol. *19*, 6297–6305.

de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M.H.A.J., and Thomma, B.P.H.J. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science *329*, 953–955.

Duan, Q., Kita, D., Li, C., Cheung, A.Y., and Wu, H.M. (2010). FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development. Proc. Natl. Acad. Sci. USA *107*, 17821–17826.

Fujiwara, M., Hamada, S., Hiratsuka, M., Fukao, Y., Kawasaki, T., and Shimamoto, K. (2009). Proteome analysis of detergent-resistant membranes (DRMs) associated with OsRac1-mediated innate immunity in rice. Plant Cell Physiol. *50*, 1191–1200.

Gómez-Gómez, L., and Boller, T. (2002). Flagellin perception: a paradigm for innate immunity. Trends Plant Sci. *7*, 251–256.

Gust, A.A., Willmann, R., Desaki, Y., Grabherr, H.M., and Nürnberger, T. (2012). Plant LysM proteins: modules mediating symbiosis and immunity. Trends Plant Sci. *17*, 495–502.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell *9*, 789–798.

Iizasa, E., Mitsutomi, M., and Nagano, Y. (2010). Direct binding of a plant LysM receptor-like kinase, LysM RLK1/CERK1, to chitin *in vitro*. J. Biol. Chem. *285*, 2996–3004.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature *444*, 323–329.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc. Natl. Acad. Sci. USA *103*, 11086–11091.

Kawano, Y., Akamatsu, A., Hayashi, K., Housen, Y., Okuda, J., Yao, A., Nakashima, A., Takahashi, H., Yoshida, H., Wong, H.L., et al. (2010a). Activation of a Rac GTPase by the NLR family disease resistance protein Pit plays a critical role in rice innate immunity. Cell Host Microbe *7*, 362–375.

Kawano, Y., Chen, L., and Shimamoto, K. (2010b). The Function of Rac Small GTPase and Associated Proteins in Rice Innate Immunity. Rice *3*, 112–121.

Kawasaki, T., Henmi, K., Ono, E., Hatakeyama, S., Iwano, M., Satoh, H., and Shimamoto, K. (1999). The small GTP-binding protein rac is a regulator of cell death in plants. Proc. Natl. Acad. Sci. USA *96*, 10922–10926.

Kawasaki, T., Koita, H., Nakatsubo, T., Hasegawa, K., Wakabayashi, K., Takahashi, H., Umemura, K., Umezawa, T., and Shimamoto, K. (2006). Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. Proc. Natl. Acad. Sci. USA *103*, 230–235.

Kim, S.H., Oikawa, T., Kyozuka, J., Wong, H.L., Umemura, K., Kishi-Kaboshi, M., Takahashi, A., Kawano, Y., Kawasaki, T., and Shimamoto, K. (2012). The bHLH Rac Immunity1 (RAI1) Is Activated by OsRac1 via OsMAPK3 and OsMAPK6 in Rice Immunity. Plant Cell Physiol. *53*, 740–754.

Kishi-Kaboshi, M., Okada, K., Kurimoto, L., Murakami, S., Umezawa, T., Shibuya, N., Yamane, H., Miyao, A., Takatsuji, H., Takahashi, A., and Hirochika, H. (2010). A rice fungal MAMP-responsive MAPK cascade regulates metabolic flow to antimicrobial metabolite synthesis. Plant J. *63*, 599–612.

Lee, S.W., Han, S.W., Sririyanum, M., Park, C.J., Seo, Y.S., and Ronald, P.C. (2009). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. Science *326*, 850–853.

Lieberherr, D., Thao, N.P., Nakashima, A., Umemura, K., Kawasaki, T., and Shimamoto, K. (2005). A sphingolipid elicitor-inducible mitogen-activated protein kinase is regulated by the small GTPase OsRac1 and heterotrimeric G-protein in rice 1[w]. Plant Physiol. *138*, 1644–1652.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., et al. (2012). Chitin-induced dimerization activates a plant immune receptor. Science *336*, 1160–1164.

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K.E., Thomma, B.P.H.J., and Rudd, J.J. (2011). Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. Plant Physiol. *156*, 756–769.

Mentlak, T.A., Kombrink, A., Shinya, T., Ryder, L.S., Otomo, I., Saitoh, H., Terauchi, R., Nishizawa, Y., Shibuya, N., Thomma, B.P.H.J., and Talbot, N.J. (2012). Effector-mediated suppression of chitin-triggered immunity by *magnaporthe oryzae* is necessary for rice blast disease. Plant Cell *24*, 322–335.

Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. (2001). Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. Nature *411*, 1065–1068.

Nakamura, T., Aoki, K., and Matsuda, M. (2005). Monitoring spatio-temporal regulation of Ras and Rho GTPase with GFP-based FRET probes. Methods *37*, 146–153.

Nakashima, A., Chen, L., Thao, N.P., Fujiwara, M., Wong, H.L., Kuwano, M., Umemura, K., Shirasu, K., Kawasaki, T., and Shimamoto, K. (2008). RACK1 functions in rice innate immunity by interacting with the Rac1 immune complex. Plant Cell *20*, 2265–2279.

Ono, E., Wong, H.L., Kawasaki, T., Hasegawa, M., Kodama, O., and Shimamoto, K. (2001). Essential role of the small GTPase Rac in disease resistance of rice. Proc. Natl. Acad. Sci. USA *98*, 759–764.

Petutschnig, E.K., Jones, A.M.E., Serazetdinova, L., Lipka, U., and Lipka, V. (2010). The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. J. Biol. Chem. *285*, 28902–28911.

Schulze, B., Mentzel, T., Jehle, A.K., Mueller, K., Beeler, S., Boller, T., Felix, G., and Chinchilla, D. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. J. Biol. Chem. *285*, 9444–9451.

Schwessinger, B., and Ronald, P.C. (2012). Plant innate immunity: perception of conserved microbial signatures. Annu. Rev. Plant Biol. *63*, 451–482.

Segonzac, C., and Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria. Curr. Opin. Microbiol. *14*, 54–61.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. Plant J. *64*, 204–214.

Shinya, T., Motoyama, N., Ikeda, A., Wada, M., Kamiya, K., Hayafune, M., Kaku, H., and Shibuya, N. (2012). Functional characterization of CEBiP and CERK1 homologs in arabidopsis and rice reveals the presence of different chitin receptor systems in plants. Plant Cell Physiol. *53*, 1696–1706.

Sorkin, A., McClure, M., Huang, F., and Carter, R. (2000). Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. Curr. Biol. *10*, 1395–1398.

Suharsono, U., Fujisawa, Y., Kawasaki, T., Iwasaki, Y., Satoh, H., and Shimamoto, K. (2002). The heterotrimeric G protein alpha subunit acts upstream of the small GTPase Rac in disease resistance of rice. Proc. Natl. Acad. Sci. USA *99*, 13307–13312.

Takai, R., Isogai, A., Takayama, S., and Che, F.S. (2008). Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice. Mol. Plant Microbe Interact. *21*, 1635–1642.

Takeuchi, M., Ueda, T., Sato, K., Abe, H., Nagata, T., and Nakano, A. (2000). A dominant negative mutant of sar1 GTPase inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus in tobacco and Arabidopsis cultured cells. Plant J. *23*, 517–525.

Thao, N.P., Chen, L., Nakashima, A., Hara, S., Umemura, K., Takahashi, A., Shirasu, K., Kawasaki, T., and Shimamoto, K. (2007). RAR1 and HSP90 form a complex with Rac/Rop GTPase and function in innate-immune responses in rice. Plant Cell *19*, 4035–4045.

Thomas, C., Fricke, I., Scrima, A., Berken, A., and Wittinghofer, A. (2007). Structural evidence for a common intermediate in small G protein-GEF reactions. Mol. Cell *25*, 141–149.

Umemura, K., Ogawa, N., Yamauchi, T., Iwata, M., Shimura, M., and Koga, J. (2000). Cerebroside elicitors found in diverse phytopathogens activate defense responses in rice plants. Plant Cell Physiol. *41*, 676–683.

Wan, J., Tanaka, K., Zhang, X.C., Son, G.H., Brechenmacher, L., Nguyen, T.H., and Stacey, G. (2012). LYK4, a lysin motif receptor-like kinase, is important for chitin signaling and plant innate immunity in Arabidopsis. Plant Physiol. *160*, 396–406.

Willmann, R., Lajunen, H.M., Erbs, G., Newman, M.A., Kolb, D., Tsuda, K., Katagiri, F., Fliegmann, J., Bono, J.J., Cullimore, J.V., et al. (2011). *Arabidopsis* lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. Proc. Natl. Acad. Sci. USA *108*, 19824–19829.

Wong, H.L., Sakamoto, T., Kawasaki, T., Umemura, K., and Shimamoto, K. (2004). Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol. *135*, 1447–1456.

Wong, H.L., Pinontoan, R., Hayashi, K., Tabata, R., Yaeno, T., Hasegawa, K., Kojima, C., Yoshioka, H., Iba, K., Kawasaki, T., and Shimamoto, K. (2007). Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell *19*, 4022–4034.

Yamaguchi, K., Imai, K., Akamatsu, A., Mihashi, M., Hayashi, N., Shimamoto, K., and Kawasaki, T. (2012). SWAP70 functions as a Rac/Rop guanine nucleotide-exchange factor in rice. Plant J. *70*, 389–397.

Yamaguchi, K., Yamada, K., Ishikawa, K., Yoshimura, S., Hayashi, N., Uchihashi, K., Ishihama, N., Kishi-Kaboshi, M., Takahashi, A., Tsuge, S., et al. (2013). A receptor-like cytoplasmic kinase targeted by a plant pathogen effector is directly phosphorylated by the chitin receptor and mediates rice immunity. Cell Host Microbe *13*, 347–357.

Yang, Z., and Fu, Y. (2007). ROP/RAC GTPase signaling. Curr. Opin. Plant Biol. *10*, 490–494.

Zhang, Y., and McCormick, S. (2007). A distinct mechanism regulating a pollen-specific guanine nucleotide exchange factor for the small GTPase Rop in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA *104*, 18830–18835.